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An automated enantioselective isolation system for the study of estrogenic potencies: Study of the estrogenic activity of α-hexachlorocyclohexane

Herein we describe an automated enantioselective isolation system that uses a gas chromatograph-flame ionization detector (FID)-preparative fraction collector to separate the enantiomers of chiral compounds. In this system, a switching device was coupled with six glass tubes containing an organic solvent for the separate collection of six compounds, thus eliminating the need for a manual collection procedure after separation by gas chromatography. To evaluate the usefulness of this system, we applied it to the separation and collection of the enantiomers of α-hexachlorocyclohexane (α-HCH), and we measured the estrogenic potency of each enantiomer by means of an MVLN cell (Michigan Cancer Foundation-7 (MCF-7) cell stably transfected with a pVit-tk-LUC reporter plasmid) bioassay and an estrogen receptor binding assay. The variability in the retention time of each enantiomer (relative standard deviation, $n = 16$) was typically <0.05%. The recoveries of the dextrorotatory (+) and levorotatory (−) enantiomers of α-HCH were about 68.8% and 73.4%, respectively. The overall quantity of each enantiomer of α-HCH collected was about 300 μg. Both enantiomers of α-HCH showed similar estrogenic activity. On the basis of the results obtained for α-HCH, we concluded that this analytical method is suitable for the determination of the estrogenic activity of other chiral compounds. Such determinations are essential because enantiomers differ considerably in how they accumulate in organisms and decompose in the environment.

Key Words: Enantioselective isolation; Gas chromatograph-preparative fraction collector; Enantiomer; Chiral compounds; Estrogenic potency

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1 Introduction

During the last decade, it has gradually become clear that the dextrorotatory (+) and levorotatory (−) isomers of chiral chemicals have different fates and effects [1, 2]. The chirality of dextrorotatory (+) and levorotatory (−) isomers of chemicals have a very real meaning for the organisms. For example, the *R* enantiomer of thalidomide, the famous drug that caused large-scale tragedy when it was used as a sleeping aid for pregnant women, is an effective medicine, whereas the *S* enantiomer is teratogenic. Like drugs, chiral pesticides are also usually enantioselective in their interactions with naturally occurring biological molecules [1]. The enantioselective analysis of chiral compounds is important for the study of their microbial catalyzed transformations, uptake into organisms, and metabolism in various organs.

Enantioselectivity in the environment was observed in many studies published in the 1990s. For example, considerable variability in the enantiomeric ratio [+/−] of α-hexachlorocyclohexane (α-HCH) in water samples taken at various stations in the North Sea was observed in 1991 [3]. HCH is a widely used organochlorine compound and is found all over the world [4]. The enantiomeric ratio [+/−] of α-HCH has been measured in many other environmental samples, and different ratios have been observed in samples taken from air [5]; water, fish, and birds [6]; and sea mammals [7, 8]. Considerable diversity in the enantioselectivity of various enzymatic degradation pathways for α-HCH has also been reported [9]. Certain HCH isomers cause central nervous system, reproductive, and endocrine damage; and the differential toxicities and environmental fates of HCH isomers have been reviewed [10–12]. The fates and effects of other chiral chemicals have also been investigated: for example, chlordane in aquatic biota [13], toxaphene in fish and seals [14], *o,p'*-dichlorodi-

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Abbreviations: α-Hexachlorocyclohexane, α-HCH; Gas chromatograph-preparative fraction collector, GC-PFC; *o,p'*-Dichlorodiphenyltrichloroethane, *o,p'*-DDT; Estrogen receptor, ER; Flame ionization detector, FID; 17β-Estradiol, E_2; Michigan Cancer Foundation, MCF.

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phenyltrichloroethane (*o,p'*-DDT) in soils [15], and polychlorinated biphenyls in shark liver [16]. Enantioselective preparative separation studies have been performed using multiple cycle approaches in order to obtain sufficient amounts for determining toxic effects and/or the absolute structures of chiral xenobiotics [17, 18].

Endocrine-disrupting compounds have become a worldwide issue, and much pertinent research has been performed [19–22]. Organochlorine compounds such as synthetic pesticides (for example, *o,p'*-DDT) would be causal compounds for endocrine disruption. Twenty-five percent of the pesticides manufactured in 1995 were chiral compounds [23]. Chlorinated chiral pesticides are almost always manufactured as their racemic mixtures. Although many of the chemicals suspected of having endocrine-disrupting potency are chiral, many of the studies of these chemicals deal only with their racemates. The *R*(−) enantiomer of *o,p'*-DDT exhibits higher estrogenic potency than the *S*(+) enantiomer in the MVLN cell (Michigan Cancer Foundation-7 (MCF-7) cell stably transfected with a pVit-tk-LUC reporter plasmid) bioassay [24]. Furthermore, individual nonylphenol congeners isolated from technical mixtures have different estrogenic potencies [25]. Thus, determining the estrogenic potencies of separated enantiomers may be essential for the study of the estrogenic effects of pollutants on organisms. However, the enantiomers of many compounds have not yet been commercially distributed.

We describe here the results of the enantiomeric analysis of the estrogenic potency of α-HCH, an analysis that involves a separation and collection procedure enabled by a switching device coupled with a gas chromatograph-preparative fraction collector (GC-PFC) system. We optimized the conditions for the separation and collection procedure, carried out the automatic collection of the dextrorotatory (+) and levorotatory (−) enantiomers of α-HCH, and evaluated the efficiency of the separate collection of the enantiomers. The GC-PFC method enabled us to get sufficient amounts (about 300 μg) of the two enantiomers to analyze their estrogenic potencies by means of two in vitro assays: an MVLN cell bioassay and an estrogen receptor (ER) binding assay.

2 Materials and methods

2.1 Chemicals

Pesticide residue-grade solvents and chemicals were obtained from Wako Chemical Co. Inc. (Tokyo, Japan). The α-, β-, γ-, and δ-HCH standards were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany) (purity: α-HCH; 97.5%, β-HCH; 97.0%, γ-HCH; 99.4%, δ-HCH; 98.4%). The working standard solutions of α-, β-, γ-, and δ-HCH were prepared in pesticide residue-grade *n*-hexane (about 420 μg/mL).

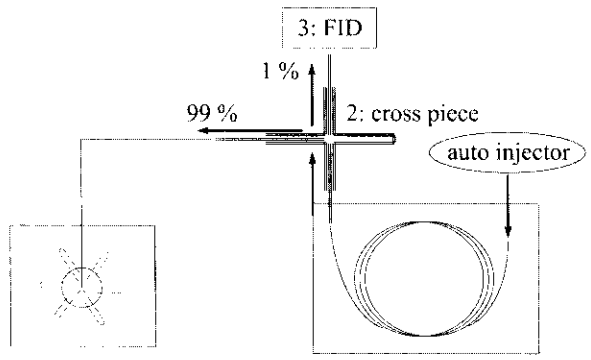

Figure 1. Schematic diagram of the gas chromatograph-preparative fraction collector system: (1) gas chromatograph with β-DEX 325 column; (2) cross piece; (3) flame ionization detector; (4) switching device: six-tube collector with cooler.

2.2 Instrumentation

A schematic diagram of the GC-PFC system (Gerstel GmbH & Co. KG, Berlin, Germany) is shown in **Figure 1**. This separation and collection system is composed of an autoinjector device, a GC, a crosspiece that separates the injected sample (1% to the detector, 99% to the collector), a flame ionization detector (FID), and a collecting device. The collecting device, controlled automatically, is composed of a switching device equipped with a cooler; the switching device automatically directs the separated sample to six glass tubes (Pyrex, volume: 100 μL). Gas chromatographic separation of α-HCH on chiral stationary phases containing cyclodextrin has been reported previously [26, 27]. In this study, we used a 30-m β-DEX 325 capillary column (0.53-mm ID, 0.5-μm film thickness, 25% 2,3-di-*O*-methyl-6-*O*-(*tert*-butyldimethylsilyl)-β-cyclodextrin embedded in SPB-20 (poly[20% phenyl/80% dimethylsiloxane]); Supelco Japan, Tokyo, Japan). To prevent the mixing of the enantiomers after GC separation, we monitored the retention time of each enantiomer by means of the FID. The typical time window of the chromatographic cut of the dextrorotatory enantiomer ranged from 28.500 to 28.919 min, and that of the levorotatory enantiomer from 29.081 to 29.500 min.

2.3 Separation and collection of the α-HCH enantiomers

We checked the suitability of this system for the separation and quantitative analysis of the estrogenic potency of chiral compounds by analyzing the separated enantiomers of α-HCH. First, the racemate standard of α-HCH dissolved in *n*-hexane (about 420 μg/mL) was prepared, and 5 μL of this solution was injected into the GC separation column with a gas-tight syringe set in an autoinjector device. The injection port temperature was programmed to hold at a starting temperature of 40°C for 1 min and then increase by

10 K/min to a final temperature of 260°C, which was held for 10 min. The GC oven temperature was programmed to hold at a starting temperature of 175°C for 3 min and then increase by 1 K/min to a final temperature of 200°C, which was held for 5 min. The transfer line (capillary column was included in the inner side) between the cross piece and the switching device was kept at 200°C. The other temperature settings were as follows: switching device temperature, 260°C; FID temperature, 280°C.

The dextrorotatory (+) and levorotatory (−) enantiomers of α-HCH were stored separately in the glass tubes with *iso*-octane (about 50 μL) cooled in an ice bath. To obtain enough material to measure the estrogenic potencies of the two enantiomers with the MVLN cell bioassay and the estrogen receptor binding assay, the separation and collection procedure was automatically repeated 400 times. The concentration and purity of each enantiomer were confirmed by GC-MS (GC, HP 5890 series II; MS, HP 5972 MSD) using selected ion monitoring (*m/z*: 181, dwell time: 100 ms, ion source temperature: 150°C, interface temperature: 280°C). Calculation of the concentration of each peak was based on the peak area of the individual enantiomer in relation to the peak area of the α-HCH racemate. We checked the blank levels of the compounds investigated. No peak was observed. The purity of each separated enantiomer (>95.8%) was calculated using the detection limit of α-HCH.

The dextrorotatory (+) and levorotatory (−) enantiomers of α-HCH were identified by their retention times and their ion ratios using a 30-m β-DEX 120 capillary column (0.53-mm ID, 0.5-μm film thickness, 20% permethylated β-cyclodextrin in SPB-35 (poly[35% diphenyl/65% dimethylsiloxane]); Supelco Japan, Tokyo, Japan) according to methods described in the literature [28, 29].

2.4 MVLN (MCF-7 ERE-Luc) cell bioassay

To date, two isoforms of the estrogen receptor, ER-α and ER-β, have been reported [30, 31]. Although the two isoforms have similar ligand binding specificities, there are differences in the distribution and relative binding affinities that could contribute to selective actions of ER agonists and antagonists in different tissues [31]. The estrogenic potencies of the dextrorotatory (+) and levorotatory (−) enantiomers of α-HCH were measured with MVLN cells, which have only ER-α. MVLN cells are MCF-7 human breast carcinoma cells stably transfected with a luciferase reporter gene under the control of the estrogen-responsive element of the *Xenopus vitellogenin* A2 gene [32]. We also carried out the MVLN cell bioassay with the β, γ, and δ isomers of HCH to evaluate and compare their estrogenic potencies. All cells were cultured in 100 mm disposable Petri plates (Corning, NY, USA) and incubated in a humidified 5% CO_2 atmosphere maintained at 37°C. MVLN cells were grown in Dulbecco's Modified Eagle Medium with Hams F-12 nutrient mixture (Sigma D-2906, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 27.3 I.U. insulin (Sigma I-1882)/L, and 1.0 mM sodium pyruvate (Sigma). Detailed methods for the in vitro bioassay have been described elsewhere [33]. In brief, the cells were trypsinized from the plates, diluted to a concentration of about 75,000 cells/mL, and seeded into the 60 interior wells of 96-well culture ViewPlates (Packard Instruments, Meriden, CT, USA) at 250 μL per well. After the cells were incubated overnight, the test and control wells were dosed with 2.5 μL of the sample or with *iso*-octane. A luciferase assay was conducted after 72 h of exposure. A luciferase assay reagent containing a luciferin substrate was added to the wells. The plates were incubated for 10 min at 30°C and then scanned with a luminescence microplate counter (LumiCount, Packard Japan, Tokyo, Japan).

2.5 ER binding assay

We performed a competitive ER binding assay to determine the affinity of (+)-α-HCH and (−)-α-HCH for human ER-β using an ER-binding assay kit (Kanto Chemical Co., Tokyo, Japan) with a 96-well microplate. In brief, the sample was dissolved in dimethyl sulfoxide and incubated with a 17β-estradiol (E_2) and ER-β solution at 37°C for 1 h. Then the mixture was added to the wells, which were coated with anti-E_2 antibodies. After that, horseradish-peroxidase-E_2 conjugate was added to each well, and the plate was incubated at 37°C for 1 h. After the substrate was added to each well, the 96-well microplate was incubated at 37°C for 20 min in the dark. After the reaction stop solution was added, the absorbance of each well was measured. The inhibition rate of each sample was calculated as the percentage displacement in the binding of E_2 to ER-β. Assays were performed in triplicate.

3 Results and discussion

3.1 Reproducibility and efficiency of the separation and collection procedure

To estimate the possibility of the separated enantiomers mixing during the collection procedure with the switching device, we measured the range of retention times for each enantiomer of α-HCH. The reproducibility of the retention times for the two enantiomers was determined by repeating the measurements 16 times within 1 day. A typical chromatogram of the separated enantiomers is shown in **Figure 2**. The retention time of the dextrorotatory enantiomer ranged from 28.710 to 28.752 min, and that of the levorotatory enantiomer from 29.263 to 29.302 min.

The variation in the retention time of each enantiomer was typically <0.05 min (relative standard deviation, <0.05%; $n = 16$). The average of resolution of the two peaks was 1.459 (relative standard deviation, <0.06%; $n = 16$).

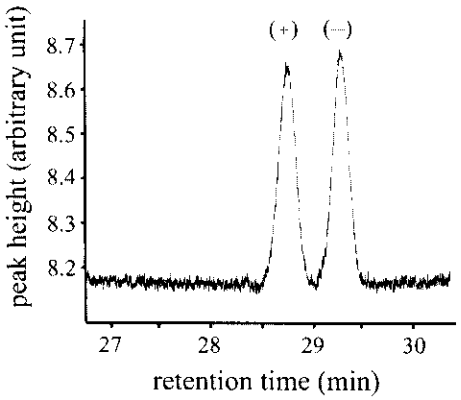

Figure 2. Typical chromatogram of α-HCH separated by means of the GC-PFC system: (+), dextrorotatory enantiomer; (−), levorotatory enantiomer. Sample injected: 2.1 μg. Calculated resolution: 1.453.

The reproducibility of the retention times remained within an acceptable range for the separation of the two enantiomers of α-HCH. We optimized the time program to yield retention times that allowed us to separate each enantiomer of α-HCH perfectly.

The efficiency of the automatic collection of each enantiomer with the switching device depends on the stability of the transfer line temperature, the switching device temperature, and the storage conditions in the glass tubes (for example, the volume of *iso*-octane and the stability of the trap temperature). To prevent temperature fluctuation in the transfer line and the switching device, we maintained a constant room temperature. The effect of the amount of *iso*-octane in the glass tube on the recovery of each enantiomer of α-HCH was also examined. The trapped amount of α-HCH was substantially enhanced when the glass tubes were filled to the halfway point with *iso*-octane. Further increases in the *iso*-octane volume led to almost no increase in the collection efficiency. The trap temperature was also varied between room temperature and 0°C. The amounts of the collected α-HCH enantiomers increased as the trap temperature was decreased from room temperature to 0°C. Decreasing the trap temperature further with liquid nitrogen brought no additional improvement in the collection efficiency. Therefore, a general trap temperature of 0°C was chosen for this study. We evaluated the recovery of the enantiomers of α-HCH by analyzing the amount of each enantiomer by GC-MS. The recoveries of the dextrorotatory (+) and levorotatory (−) enantiomers of α-HCH were about 68.8% and 73.4%, respectively.

3.2 Estrogenic potency measured with the MVLN cell bioassay

The GC-PFC system, which could be operated precisely when the time program was suitably adjusted, was used to determine whether there was a statistically significant difference in estrogenic potency between the two enantiomers of α-HCH. The relative estrogenic potencies of (+)-α-HCH, (−)-α-HCH, α-HCH (racemate), and the other HCH standards (β-, γ-, and δ-HCH) were calculated from the results of the MVLN cell bioassay according to the method described in the literature [34]. As shown in **Table 1**, higher estrogenic potencies were confirmed for γ-and δ-HCH than for α- and β-HCH. No significant difference in relative estrogenic potency was observed between (+)-α-HCH and (−)-α-HCH in the MVLN cell bioassay. Relative estrogenic potencies (%) of (+)*o,p'*-DDT and (−)*o,p'*-DDT were estimated as $<1 \times 10^{-5}$ and about 1×10^{-4}, respectively [24]. The results show that the HCH isomers are weak endocrine disruptors.

Table 1. Relative estrogenic potencies of HCH isomers using the MVLN cell bioassay.

Compound	Relative estrogenic potency (%)
α-HCH (racemate)	$2.97 \times 10^{-6} \pm 0.45 \times 10^{-6}$
(+)-α-HCH	$2.86 \times 10^{-6} \pm 0.19 \times 10^{-6}$
(−)-α-HCH	$2.72 \times 10^{-6} \pm 0.33 \times 10^{-6}$
β-HCH	$2.55 \times 10^{-6} \pm 0.21 \times 10^{-6}$
γ-HCH	$9.24 \times 10^{-5} \pm 1.30 \times 10^{-5}$
δ-HCH	$6.67 \times 10^{-5} \pm 1.00 \times 10^{-5}$

The relative estrogenic potency is the ratio between the dose of 17β-estradiol and the dose of xenobiotic needed to produce maximal luminescence intensity ×100. Assays were performed in triplicate.

3.3 Estrogen receptor-β inhibition rates

The ER binding affinity of estrogen agonists or antagonists may be related to their potencies relative to endogenous estrogen. Thus, receptor binding assays have been used to screen for potential estrogenic compounds [35]. The inhibition rates of (+)-α-HCH and (−)-α-HCH in the ER-β binding affinity assay are shown in **Figure 3**. Unlike the MVLN cell bioassay, the (−)-α-HCH was found be significantly more potent than the (+)-α-HCH in inhibition rate of ER-β binding affinity assay below a concentration of 100 μM. The significant difference between (+)-α-HCH and (−)-α-HCH was reported concerning the cytotoxic effect as well as the growth stimulation of mitosis in primary rat hepatocytes [10]. Thus, the quantitative determination of α-HCH as racemic mixture may lead to an overestimation or underestimation of the toxicity of this compound and the enantioselective determination of the potencies of α-HCH may be essential for the study of the biological effects on organisms.

4 Concluding remarks

We have described the separation of the enantiomers of α-HCH with an automated isolation device that uses a GC-PFC system. The estrogenic potencies of the indivi-

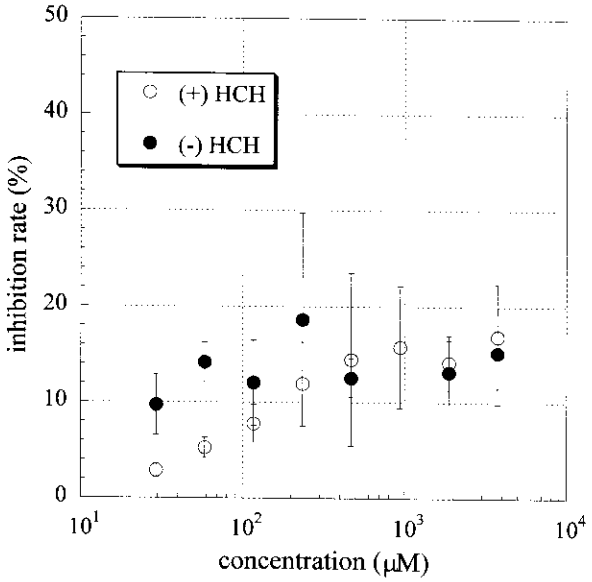

Figure 3. Inhibition rate of (+)-α-HCH and (−)-α-HCH in the estrogen receptor-β binding affinity assay. Assays were performed in triplicate.

dual enantiomers were analyzed by means of two sensitive in vitro bioassays. We believe that this system will allow researchers to determine the potency of the dextrorotatory (+) and levorotatory (−) enantiomers of chiral estrogenic compounds in the environment and to assess the fate and toxicity of numerous other chiral compounds.

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Determination of drugs used in advanced breast cancer by capillary gas chromatography of pharmaceutical formulations

A capillary gas chromatographic method with flame ionisation detection (FID) has been developed for the analysis of Tamoxifen, Anastrozole, and Letrozole in their pharmaceutical preparations, using Clomipramine as the internal standard in order to achieve quantification. Optimal conditions were investigated: column head pressure (110 kPa), injector and detector (FID) temperatures (both 325°C), time and temperature for the splitless step (0.75 min and 70°C), volume injected (2 μL), and an oven temperature program providing analysis times shorter than 8 minutes. The principal aspects of the validation method are examined and discussed. A test of the ruggedness of this method was carried out using the Plackett-Burman fractional factorial model with a matrix of fifteen experiments. Detection limits of 17.0, 9.6, and 30.6 μg L^{-1} were obtained for Tamoxifen, Anastrozole, and Letrozole, respectively. This method is simple, rapid, and sensitive; it was tested on the analysis of pharmaceutical preparations, achieving recoveries between 98.8% and 100.3%.

Key Words: Tamoxifen; Anastrozole; Letrozole; Pharmaceutical products; Breast cancer; Capillary gas chromatography

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1 Introduction

Breast cancer is the most common malignancy of women in the western hemisphere. Many of these patients develop metastatic disease, for which no cure is currently available. Oestrogen is known to play a major role in the development and proliferation of breast cancer. Although ovarian oestrogen synthesis ceases at the menopause, oestrogen production still takes place in peripheral tissues by the aromatisation of circulating androgens [1]. The main pathway for oestrogen formation is the conversion of androstenedione to oestrone, with a smaller contribution from the conversion of testosterone to oestradiol [2].

Patients with hormone receptor-positive tumours are treated with endocrine therapies such as the anti-oestrogens, which inhibit oestrogen activity at the cellular level, or with aromatase inhibitors, which inhibit oestrogen production. Both treatments have the advantage of having fewer side effects than chemotherapy.

For over 20 years, the anti-oestrogen Tamoxifen (**Figure 1**) was the treatment of choice for oestrogen receptor (ER)-positive breast cancer. However, the partial oestrogen agonist properties of the drug, together with a high incidence of recurrence in patients treated with Tamoxifen, prompted the need for better treatment regimens. The introduction of the third generation aromatase inhibitors, Anastrozole and Letrozole (Figure 1), led to a reappraisal of the treatment algorithm for hormone-dependent breast cancer. Preliminary studies were promising, with both compounds demonstrating a significant suppression of plasma oestradiol levels in postmenopausal women, as well as tumour regression in a large proportion of patients [3, 4].

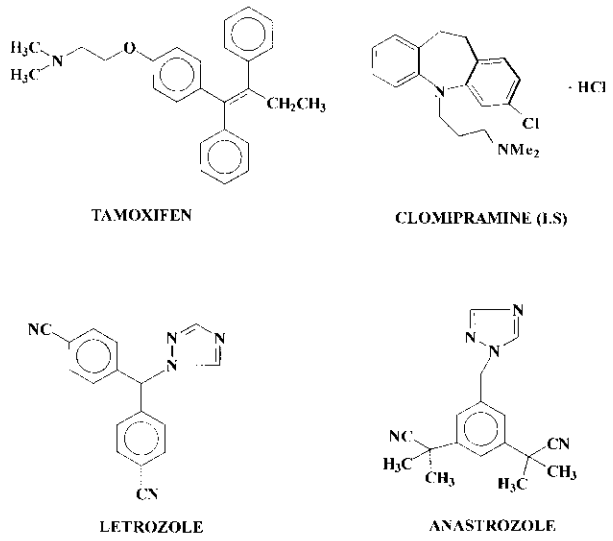

Figure 1. Structure of the molecules.

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